# ESR EVIDENCE FOR SEQUENTIAL DIVALENT CATION BINDING IN HIGHER PLANT CELL WALLS

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**Electron spin resonance linewidth measurements have been made on intact cell walls exchanged with various combinations of $Mn^{2+}$ and $Ca^{2+}$. These experiments were performed to find the $Mn^{2+}$ nearest-neighbor distance and thereby determine whether carboxylate-$Mn^{2+}$ complexes potentiate ion association at adjacent sites on cell wall polyuronides. Our results show that as the fraction of available binding sites occupied by $Mn^{2+}$ increased from 2% to 27%, the nearest-neighbor distance parameter decreased only from 14 to 11 Å. These distances are close to polyuronide interanionic spacings. The small change in the distance parameter with concentration is evidence for sequential rather than random binding. Competitive ion-exchange with $Ca^{2+}$ was found to reduce the $Mn^{2+}$ spin-spin line broadening at similar total bound $Mn^{2+}$ concentrations. This is expected only if $Ca^{2+}$ competes at adjacent sites. The data presented offer strong support for the hypothesis that carboxylate groups near already occupied sites have a greater affinity for divalent cations than other sites along the polyuronide main chain.**

## Introduction

Polyuronic acids are a major component of the primary cell wall and middle lamellae of higher plant tissues [1,2]. These unique cell wall components are efficient cation exchangers [3,4] and, therefore, can affect ion activity coefficients, transmembrane potential, and electrolyte flux [5]. The divalent cationic salts of polyuronide-containing cell wall polymers might also play a structural role in adjacent cell wall to wall adhesion.

Solution studies on polygalacturonate and polyguluronate [6–12] indicate a cooperative type of ion binding which has a molecular size dependency [8,9]. One hypothesis [7,11] proposes that certain uronide polymers bind divalent cations in such a way that the ions are ordered in electronegative cavities between chains like 'eggs in an egg box.' Little information is available concerning the ion-binding mechanism or the polyuronide-ion aggregation structure in solid matrices [3–5,13]. In this report, we present data which indicate that carboxylate-divalent cation complexes potentiate ion association at adjacent sites on cell wall polyuronides.

## Materials and Methods

Intact cortical tissue of unripe *Malus pumila* fruit was used in all experiments reported in this study. The plant tissues were fixed by dehydrating 1 h each in 20, 40, 60, 80, 95 and 100% (v/v) ethanol/water in vacuo. Cross-polarization and magic-angle spinning $^{13}C$-NMR spectra of cell

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wall material prepared in this way reveals no aliphatic or aromatic resonances, indicating a low abundance of lipids or other extraneous materials which might adhere to the cell wall. Cross-polarization and magic-angle spinning NMR spectra (8 kHz spectral width) were obtained at a $^{13}C$ frequency of 15 MHz on a JEOL FX-60QS spectrometer with a $^{1}H$ decoupling field strength of 11 G. 1K data points were sampled and zero-filled to 4K for data acquisition. All chemical shifts were assigned relative to hexamethylbenzene's methyl resonance (17.36 ppm) based on the position of trimethylsilane. Samples (300–500 mg dry wt.) were spun (2.4 kHz) at the magic angle (54.7 °C) in a Kel-F bullet rotor. Setting the angle with hexamethylbenzene was performed prior to each experiment. All cross-polarization and magic-angle spinning $^{13}C$-NMR experiments were performed in the presence of dry nitrogen gas flow. Areas under resonance peaks were determined by triangulating to the baseline and taking three planimeter measurements/area. Spectra used for quantitative measurements were acquired using 0.8 ms contact period. The quantitation of free carboxylate groups was determined by subtracting the areas of the methyl ester resonance ($\delta$ 54 ppm) from the total carboxylate resonance ($\delta$ 172 ppm) and dividing this value by the anomeric carbon resonance ($\delta$ 105 ppm) area. Since the anomeric carbon signal is a measure of all cell wall carbohydrates (e.g., one anomeric carbon per carbohydrate monomer), this ratio reflects the fraction of unmethylated uronide polymers in these materials. From this knowledge it is easy to calculate the uronic acid content on a mol/g cell wall basis.

For ESR experiments, intact tissues ($1 \times 1 \times 4$ mm) were equilibrated in $H_2O$ (pH 7) for 15 min and decanted, and this was repeated twice. Approx. 40 mg (dry weight equivalent) of cell wall were used for each ion-exchange treatment. These samples were equilibrated ($24 \pm 2$ °C) in 15 ml (pH 5.2; cell wall uronide $pK_a = 3.2$ [14]) of various combinations of $MnSO_4$ ($10^{-4}$–$10^{-2}$ M) and $CaCl_2$ (0–$10^{-2}$ M). After about 22 h, the solutions were decanted and the specimens washed thrice in water (pH 7), allowing 15 min equilibration in each wash. After an additional 1 h soak, this washing procedure was repeated. About 10 mg (dry weight equivalent) of the cell wall preparation were carefully loaded into $3 \times 130$ mm quartz ESR tubes. The remaining sample was washed with methanol, vacuum-dried (35 °C), weighed, and dry ashed for atomic absorption spectrophotometric analysis of $Mn^{2+}$ and $Ca^{2+}$ using standard procedures.

All ESR spectra were obtained on a Varian Series E-109B * spectrometer at 97 K. The experimental parameters were: scan microwave power, 20.75 dB; scan time, 8 min; time constant, range, 5 kG; field set 3224.4 G; microwave frequency, 9.115 GHz; 0.064 s. The empirical measure of line broadening (see Fig. 2, insert, for the line-broadening factor calculation) was directly proportional ($r^2 = 0.99$) to the line width of computer-generated first-derivative spectra in the line-broadening factor range 25–90, within which all our data fell.

## Results and Discussion

The results in Fig. 1 demonstrate that bound $Mn^{2+}$ is associated with an acid-titratable site. Polyuronic acid-containing cell wall components are the likely ion-binding species, since they are the predominant anionic component [2] in these tissues.

Spin-spin [15] line broadening is a dominant mechanism for linewidth alterations in $Mn^{2+}$ ESR experiments. This form of line broadening has a $1/r_{ij}^3$ dependency where $r_{ij}$ is the distance between the $i$th and $j$th paramagnetic ions. Van Vleck [17] has treated the case of a uniform powder of interacting spins and has given relation 1 for the second moment $(\Delta v^2)_{ave}$ of the resultant ESR line.

$$(\Delta v^2)_{ave}^{1/2} = \left(3/5 \cdot g^4 \beta^4 h^{-2} \cdot S(S+1) \cdot \Sigma_i 1/r_{ij}^6\right)^{1/2} \qquad (1)$$

where the units are Hz. The constants $g$, $\beta$ and $h$ have their usual values and $S$ represents the total electron spin of the ion ($S = 5/2$ for $Mn^{2+}$). We have simplified this relation to (2) making use of the fact that for a Gaussian line the linewidth ($\Delta H_{1/2}$) is twice the second moment. In this relation, $d$ is the distance (Å) between nearest neigh-

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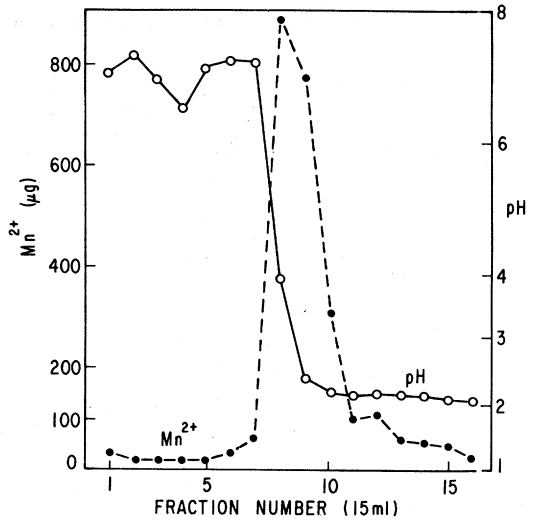


Fig. 1. Titration of $Mn^{2+}$ off intact cell wall matrices under mildly acidic conditions (ion-depleted $H_2O$ adjusted to pH 2 with 0.1 M HCl). Approx. 300 mg (dry weight equivalent) of cortical tissue were loaded into a 2×24 cm glass column, thoroughly washed with about 500 ml (pH 7) water followed by a rinse with an equivalent volume of 0.1 M $MnSO_4$ (pH 5.2; solution flow = 4 ml/min). Excess $Mn^{2+}$ was washed from the tissues with 500 ml of water (pH 7). Fractions (15 ml) were collected after 250 ml (pH 2) water had been added. Atomic absorption analysis and pH measurements were performed on each fraction. ○, pH; ●, [$Mn^{2+}$].

bors (or the lattice constant for larger arrays); $\kappa$ depends on the arrangement of ions and is 1.00 for a two-spin system, 1.42 for a linear array of spins, and 2.85 for a cubic lattice of spins.

$$\Delta\Delta H_{1/2} = 28690 \cdot G \cdot \text{Å}^3 \cdot (S(S+1))^{1/2} \cdot \kappa / d^3; \text{ (units = Gauss)} \quad (2)$$

For the purposes of this work we assume $\kappa$ is constant at 1.42 (i.e., a linear array of manganese ions). It is likely that as ion-binding sites fill, $\kappa$ will increase from 1.00 (low bound ion concentration) up to a maximum of 2.85 (high bound ion concentration). At very small separations ($d$ less than 10 Å) spin-spin exchange can result [17] in line narrowing. At the separation distances found in this work no exchange narrowing was observed. Because of this spacial dependency, changes in linewidth can be utilized to estimate the average distance between adjacent paramagnetic ions within a narrow range ($d$ less than 20 Å). Beyond 20 Å spin-spin line broadening makes no significant contribution. The nearest-neighbor distance ($d$) was estimated for cell wall-bound $Mn^{2+}$ (from Eqn. 2, $\kappa = 1.42$) by two different techniques which are described in Table I. The techniques involved (1) computer simulations of experimental spectra, and (2) direct measurement of line broadening from the experimental spectra. The greatest dis-

TABLE I

FRACTION OF CELL WALL POLYURONIDE BINDING SITES OCCUPIED BY $Mn^{2+}$, AND CORRESPONDING $Mn^{2+}$ LINE-BROADENING FACTORS (LBF) AND NEAREST-NEIGHBOR DISTANCE PARAMETERS ($d$)

Fraction of sites occupied: (mol $Mn^{2+}$/g dry weight cell wall)/([mol $COO^-$/g dry weight cell wall]/2)×100; mol $Mn^{2+}$ determined by atomic absorption spectrophotometry; mol $COO^-$ determined via cross-polarization and magic-angle spinning $^{13}C$-NMR.

$d(1)$ was determined by simulating the experimental spectra with computer-generated first-derivative spectra at various linewidths. The linewidth, which resulted in the same line-broadening factor for experimental and computer simulation, was used to calculate $d$ (see Eqn. 2 in the text). $d(2)$ was determined by directly measuring the sample incremental increase in linewidth from the experimental spectra. In this method we assumed that the increase in the total width was a measure of the line broadening of the end components. This probably exaggerates the line broadening slightly. The same relation between linewidth and nearest neighbor distance, employed for $d(1)$, was utilized here. The change in nearest-neighbor distance ($d$) may also be interpreted as a change in the constant $\kappa$ (Eqn. 2) with sites occupied at a constant value of $d$. The actual case is probably intermediate between $\kappa = 1$ (low concentration of ion bound) and $\kappa = 2.85$ (when most of the available binding sites have been filled).

For comparison purposes, the line-broadening factor of a $5 \cdot 10^{-2}$ M solution of $Mn^{2+}$ ($5 \cdot 10^{-5}$ mol $Mn^{2+}/cm^3$) in 50% glycerol/water is equal to about 26. Assuming that the density of the cell wall polyuronide fraction is equivalent to crystalline cellulose (this would necessarily by an overestimation), our value for the 2.5% sample is approx. $3.77 \cdot 10^{-5}$ mol $Mn^{2+}/cm^3$. These data are indicative of a nonrandom or sequential binding mechanism.

| Sites occupied: (%) | LBF | Distance parameter (Å) | | |
|---|---|---|---|---|
| | | $d(1)$ | $d(2)$ | Mean ± S.E. |
| 2.5 | 47 | 13.6 | 12.9 | 13.3 ± 0.5 |
| 12.9 | 67 | 12.4 | 12.0 | 12.2 ± 0.3 |
| 24.4 | 84 | 11.6 | 10.9 | 11.2 ± 0.5 |
| 27.3 | 89 | 11.4 | 10.0 | 10.7 ± 1.0 |

agreement between the three methods was only 1.4 Å; the average deviation was 0.8 Å. The data in Table I demonstrate that, at a low bound $Mn^{2+}$ concentration (about $8 \cdot 10^{-6}$–$9 \cdot 10^{-5}$ mol/g dry weight of cell wall), the average inter-paramagnetic ion distance was of the order of 11–14 Å. This value is close to polyuronide intercarboxylate spacings (6–15 Å; estimated from the 'egg box' model [11] of polyguluronate aggregation in solution or as reported [14] for adjacent carboxylate distances in *Lemna minor* cell walls, respectively). The small change in $d$ with the fraction of sites occupied suggests a nonrandom distribution of ions on binding sites. In fact this argues that paramagnetic ions preferentially associate with cell wall polyuronides in a sequential or 'zipper'-like fashion. The $Mn^{2+}$ nearest-neighbor distance parameter does diminish by as much as 2.2 Å as the fraction of available binding sites decreases, possibly due to binding at neighboring chain sites (which would change $\kappa$) or bound ion-dependent alterations in polymer conformation.

If the sequential binding hypothesis is true, competitive exchange with a nonparamagnetic ion should reverse the effect. The $Mn^{2+}$ spin-spin line broadening effect is largely lost when the cell walls are exchanged with both $Mn^{2+}$ and $Ca^{2+}$ (Fig. 2).

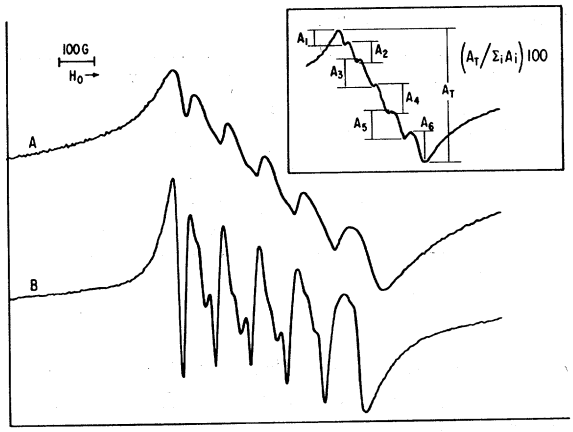


Fig. 2. ESR spectra of cell wall-absorbed $Mn^{2+}$ as a function of bound $Ca^{2+}$. The cell wall sample represented in spectrum A has about $4 \cdot 10^{-5}$ mol $Mn^{2+}$/g dry weight cell wall ($X_{Mn^{2+}} = 1$); the cell wall sample represented in spectrum B had an equivalent level of the paramagnetic ion but $X^{2+}_{Mn} = 0.3$. Inset spectrum: illustration of the experimental line-broadening factor, LBF = $((A_T/\Sigma A_i)100)$.

These spectra were obtained from samples having equivalent levels of bound $Mn^{2+}$ (about $4 \cdot 10^{-5}$ mol/g); however, spectrum B is qualitatively similar to those obtained from dilute $Mn^{2+}$ glycerol/$H_2O$ solutions at 97 K. The small hyperfine lines seen in the most well-resolved spectrum (B) are due to the effect of the crystal field [18] on the Zeeman levels; the line shape is also broadened by negligible ion reorientation rates at these near-liquid $N_2$ temperatures (i.e., homogeneous secular broadening). Spectra, with nearly identical crystal field splittings, were obtained from dilute $Mn^{2+}$ solutions at 97 K; this is evidence that the crystal field contribution to line shape is effectively the same in both cases. We find the line broadening factor which results from the $Mn^{2+}$ nearest-neighbor spin-spin interaction is greatly reduced as a function of the mole fraction of $Ca^{2+}$ bound ($X_{Ca^{2+}} = ([Ca^{2+}]\text{bound}/[Mn^{2+} + Ca^{2+}]\text{bound}$; Fig. 3). A linear relationship is observed between the nearest-neighbor distance ($d$) and $X_{Ca^{2+}}$, which might be expected, assuming a uniform sequential array of bound ions [16]. These data therefore indicate that the binding of the nonparamagnetic calcium ion to cell wall polyuronide-containing polymers is similar to that of $Mn^{2+}$.

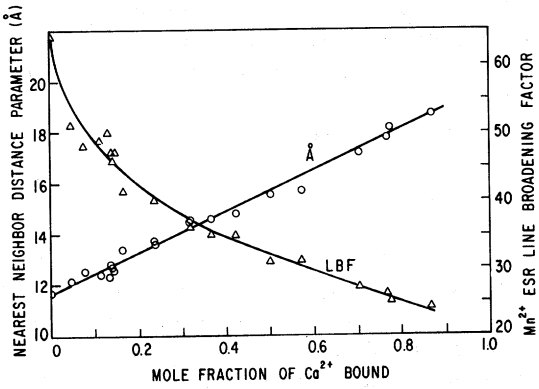


Fig. 3. Changes in linewidth as measured by the line-broadening factor (△) and calculated paramagnetic ion nearest-neighbor distance parameters ($d(1)$) (○) as a function of the mole fraction of $Ca^{2+}$ bound ($[Ca^{2+}]\text{bound}/[Ca^{2+} + Mn^{2+}]\text{bound}$). The fraction of sites filled by $Mn^{2+} = 22.4 \pm 1.5\%$ (±S.E.) for samples with line-broadening factor values between 64 and 30; the change in the factor can therefore only be associated with a competitive binding effect.

From these studies, we conclude that certain divalent cations bind to cell wall polyuronides in a spacially sequential fashion. We view this form of association as a rather special case of cooperativity whereby the probability of ion binding at sites more than about 15 Å from existing carboxylate-ion complexes is small. Furthermore, we propose that the cell wall ion-polyuronide aggregate structure is similar to that hypothesized for polyuronides in solution [7,11]. In this model, the metal ions are chelated by electrostatic and ionic forces between adjacent polymer chains. Our data indicate that the occupation of some initial site leads to an alteration in polymer structure and creates the potential for metal ions to associate with nearest-neighbor carboxylate groups. As a consequence of this cooperative effect, ion binding would proceed in a zipper-like fashion. Work is presently in progress to determine whether this mechanism is a generalized phenomenon in other anionic biopolymer systems.

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